METHEMOGLOBIN FORMATION AND RED CELL METABOLISM OF GUINEA PIGS DURING CHRONIC HYPERCAPNIA

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SUMMARY PAGE

PROBLEM

To determine the basis for decreased oxygen capacity in the blood of mammals exposed to carbon dioxide.

FINDINGS

The significant decrease in oxygen capacity of guinea pig blood (although there is no change in total hemoglobin concentration) following 7 days of exposure to 15% CO_2 results from oxidation of hemoglobin to methemoglobin. The methemoglobin not only decreases O_2 content of arterial blood, but also may result in a left shift in the oxygen dissociation curve, further compromising oxygen delivery to tissues. The methemoglobin levels during acute and chronic hypercapnia are correlated with inhibition by decreased pH of those enzyme systems which reduce methemoglobin to hemoglobin. Exposure to 3% CO_2 for seven days did not result in methemoglobin formation.

APPLICATION

The findings are important to environmental life scientists, submarine medical officers, and others who deal with closed environmental systems in which CO_2 (or other factors, e.g. OHP) may alter erythrocyte metabolism resulting in methemoglobin production.

ADMINISTRATIVE INFORMATION

This investigation was conducted as a part of Bureau of Medicine and Surgery Research Work Unit M4306.02-2030BAK9 - Interaction of Carbon Dioxide with O_2 and Inert Gases at High Pressure During Naval Diving Operations. The present report is No. 1 on this Work Unit. It was approved for publication on 3 May 1971 and designated as Submarine Medical Research Laboratory Report No. 665. During this period in which the investigation was conducted, Stephen C. Wood held a National Research Council - Bureau of Medicine and Surgery Postdoctoral Research Associateship.

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ABSTRACT

Recent studies of chronic hypercapnia in guinea pigs and rats demonstrated a biphasic shift in the blood oxygen dissociation curve, a diminution of heme-heme interaction and a significant decrease in the oxygen capacity of blood which was not due to decreased hemoglobin content. The latter finding suggested production of non-functional hemoglobin during hypercapnia. We measured the concentration of oxidized (met) hemoglobin and found an increase from control values of 2.69% to 8.73% at 1 day, 6.4% at 3 days and 6.2% at 7 days of 15% CO_2 exposure. The values are correlated with the biphasic changes in oxygen affinity (P_{50}) and heme-heme interaction (n) of blood oxygen dissociation curves during hypercapnia. Hemoglobin oxidation is enhanced by low pH, and inhibition of red cell catalase by CO_2 resulting in increased oxidation by H_2O_2 may also be partially responsible for methemoglobin formation. The persistence of methemoglobin results from inhibition by low pH of the enzyme systems which convert methemoglobin to hemoglobin.

METHEMOGLOBIN FORMATION AND RED CELL METABOLISM OF GUINEA PIGS DURING CHRONIC HYPERCAPNIA

INTRODUCTION

Recent studies of oxygen transport properties of guinea pig blood during chronic hypercapnia have shown the following: A biphasic shift of the oxygen dissociation curve which parallels the eyrthrocyte pH changes[20]; a pH-dependent change in the red cell 2,3-diphosphoglycerate (DPG) concentration[15]; an increase in total blood volume due to increased red cell volume[1]; a significant decrease in the oxygen capacity of blood[20]. The apparent paradox of the last two findings prompted us to examine the levels of methemoglobin and other non-functional hemoglobin derivatives during chronic hypercapnia. We also examined catalase activity, reduced glutathione (GSH) concentration and glucose-6-phosphate dehydrogenase (G-6-P D) activity. Data on other aspects of red cell metabolism related to the maintenance of hemoglobin iron in the ferrous form were provided by separate investigations in this laboratory which measured levels of reduced nicotinamide adenine dinucleotide (NADH)[9] and phosphofructokinase activity[10] of red cells during hypercapnia.

MATERIALS AND METHODS

Studies were performed on male guinea pigs (Hartley strain) weighing between 400 and 600 gm. Chronic hypercapnia was induced by exposing the animals to 3% of 15% CO_2 in air for periods up to seven days. Exposure took place in temperature-controlled environmental chambers. Criteria for selecting experimental animals and details of the exposure procedure are described by Schaefer, McCabe and Withers[21]. Blood samples were obtained in heparinized syringes from the abdominal aorta of anesthetized animals (pentobarbital, 40 mg/kg ip) which were breathing air (controls), 3% CO_2 or 15% CO_2 through a face mask. Samples were obtained after 6 hours, 1 day, 3 days and 7 days of CO_2 exposure and analyses were done immediately.

Methemoglobin, carboxyhemoglobin and sulfhemoglobin concentrations were measured with a Cary Model 14 spectrophotometer according to the methods of Dubowski[4]. The precision of the methemoglobin determination in our laboratory was $\pm$ 0.1 g methemoglobin per 100 ml blood. Reduced glutathione (GSH) was determined by a modification of the nitroprusside method[2]. Glucose-6-phosphate dehydrogenase activity was determined using Boehringer reagents according to the method of Lohr and Waller[14].

Blood pH was determined with an IL Model 113 pH analyzer. Values were corrected to the measured body temperature[22].

Student's "t" test was used for statistical evaluation of the data.

RESULTS

The results of the methemoglobin, GSH and glucose-6-phosphate dehydro-

genase activity determinations are summarized in Table 1. Carboxy- and sulfhemoglobin formation did not occur in any experimental animal. The methemoglobin concentration increased to a peak value at one day and returned to lower values at 3 and 7 days but remained significantly higher than the control value. Glutathione concentration and G-6-P D activity, on the other hand, remained relatively constant until the seventh day of hypercapnia when both decreased significantly. Figure 1 presents the time course of changes in

TABLE I - Effect of chronic hypercapnia on methemoglobin concentration, reduced glutathione concentration and glucose-6-phosphate dehydrogenase activity of guinea pig erythrocytes.

Condition		Methemoglobin % of total Hb	GSH mg/100 ml red cells	G-6-P D mUnits/ml red cells
Control	Mean	2.69	123.2	111
	SEM	0.55	4.3	6
	N	7	4	32
15% CO_2				
6 hours	Mean	3.54	124.1	91
	SEM	0.67	8.1	5
	N	8	4	14
1 day	Mean	8.73*	112.9	100
	SEM	0.59	5.6	3
	N	7	4	16
3 days	Mean	6.40*	126.2	103
	SEM	0.83	8.7	12
	N	7	4	10
7 days	Mean	6.20*	86.6*	63*
	SEM	0.83	5.3	6
	N	6	4	5

* Statistically significant at 5% level or better.

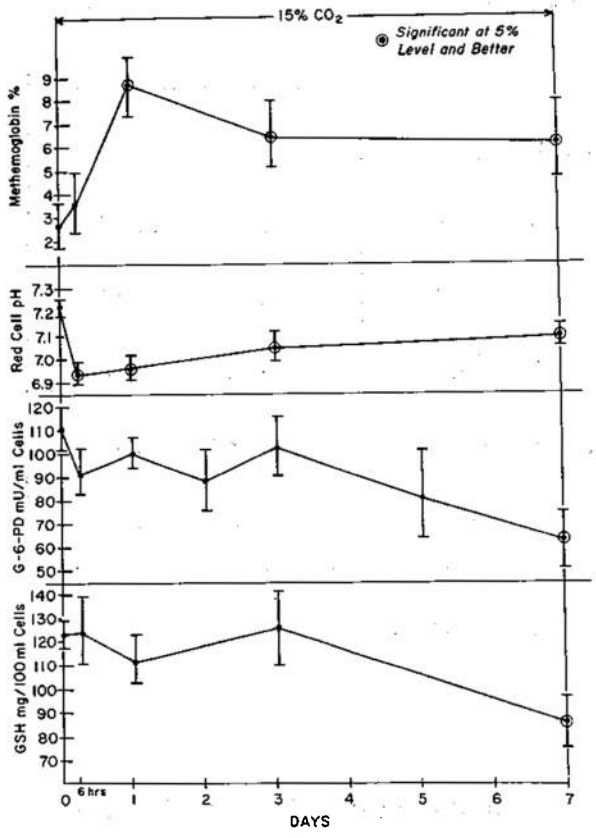

Fig. 1. The course of changes in methemoglobin concentration, red cell pH, Glucose-6-phosphate dehydrogenase activity, and reduced glutathione concentration during chronic hypercapnia. Red cell pH data from Schaefer, et al. (1). Vertical bars represent mean ± 2 SE's. Significance at $P < 0.05$ is indicated if vertical bars for different values do not overlap.

these parameters and compares these values with changes in red cell pH. The biphasic change in methemoglobin concentration corresponds closely with red cell pH but is clearly not directly related to the GSH concentration or G-6-P D activity.

In order to extend these observations, we also exposed guinea pigs to 3% CO_2 in air for 1 hour, 6 hours, 1 day, 3 days and 7 days. It had been previously established that blood pH after 1 day of 3% CO_2 corresponds closely to the blood pH after 7 days of 15% CO_2 (Schaefer, unpublished). The present results, given in Table II, confirm this significant but mild acidosis during the acute phase, but there was no statistically significant change in methemoglobin level.

DISCUSSION

The degree of methemoglobin formation during hypercapnia accounts quantitatively for the decreased O_2 capacity reported by Schaefer, et al.[20]. They found that the O_2 capacity decreased from a mean control value of 16.8 vol% to 14.8 vol% after 7 days of 15% CO_2 exposure. Using their values for hemoglobin concentration and the Hüfner factor of 1.34 ml O_2/g Hb, the observed concentration of 6% methemoglobin would account for this diminished O_2 capacity.

The correlation between the time course of methemoglobin concentration in the present study and the oxygen affinity (P_{50}) of whole blood dissociation curves reported by Schaefer, et al.[20] deserves further consideration. Although there is convincing evidence that the biphasic change in P_{50} results from biphasic changes in erythrocyte 2,3-

TABLE II - Effect of 3% CO_2 on Blood pH and Methemoglobin Level in Guinea Pigs

Condition	N	Blood pH $\bar{x} \pm SE$	Met Hb of total $\bar{x} \pm SE$
Control	5	7.407 ± .020	2.69 ± 0.55
3% CO_2 1 hour	5	7.274 ± .031*	3.04 ± 0.19
3% CO_2 6 hours	5	7.287 ± .020*	2.78 ± 0.49
3% CO_2 1 day	5	7.305 ± .0067*	3.24 ± 0.30
3% CO_2 3 days	6	7.337 ± .013*	2.87 ± 0.36
3% CO_2 7 days	6	7.354 ± .012	2.82 ± 0.40

* Significant at $P<.05$ level.

diphosphoglycerate concentration[15], the correlation between methemoglobin concentration and P_{50} during hypercapnia is compatible with previous reports that methemoglobin decreases the P_{50} of blood in vitro and in vivo[3,7]. There is also evidence that heme-heme interaction ("n" values of the Hill equation) of oxygen dissociation curves is decreased in the presence of methemoglobin[3]. This is supported by the data of Schaefer, et al.[20] who found that the "n" value decreased from 2.874 for controls to 2.464 after 1 day of 15% CO_2 exposure and returned to 2.592 at 3 days and 2.641 at 7 days. These values are closely correlated with the methemoglobin concentrations found in the present study ($r=0.99$ $P<0.001$).

Possible mechanisms of CO_2 involvement in hemoglobin oxidation and methemoglobin reduction are shown schematically in Figure 2. The principal function of red cell metabolism, aside from ATP production for "pump" operation, is the generation of reducing potential to protect enzymes, hemoglobin and membrane proteins against oxidation. The intermediate of anaerobic glycolysis (F-6-P → Pyruvate in Figure 2) providing this reducing potential is NADH while the hexose shunt (G-6-P → 6PG in Figure 2) provides both GSH and reduced nicotinamide adenine dinucleotide phosphate (NADPH).

Since methemoglobin is the thermodynamically stable form when oxygen is

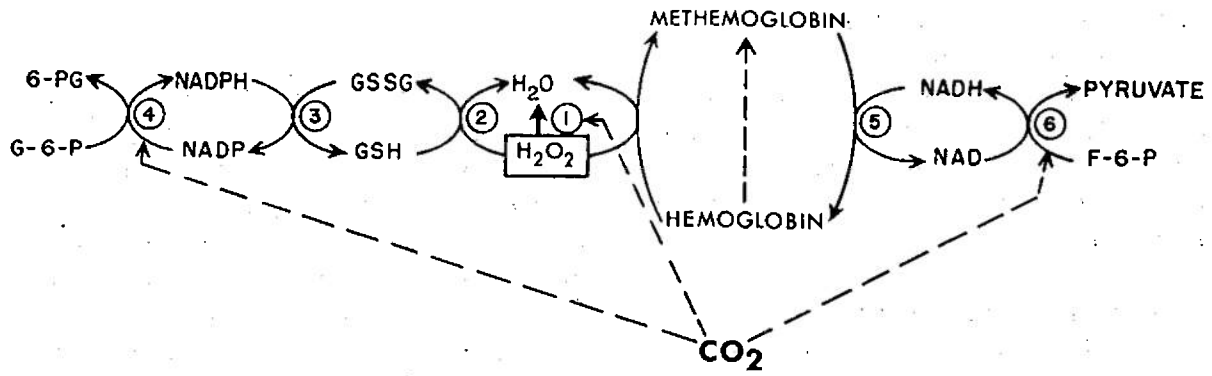

Fig. 2. Schematic representation of the steps in red cell metabolism involved in protection of hemoglobin from oxidation by hydrogen peroxide, reduction of methemoglobin to hemoglobin, and the points of interference by CO_2 (or pH). (1) catalase, (2) GSH peroxidase, (3) GSH reductase, (4) G–6–P Dehydrogenase, (5) NADH dependent methemoglobin reductase, (6) phosphofructokinase. Dashed lines from CO_2 indicate enzymes which are inhibited by CO_2. Dashed line from hemoglobin indicates autoxidation or oxidation by indirect oxidants. See text for discussion.

present[8], hemoglobin is susceptible to autoxidation and to coupled or direct oxidation by a wide variety of agents. We first thought that oxidation by hydrogen peroxide was a likely candidate in the present experiment since it has been shown that CO_2 is a potent inhibitor (independent of pH) of erythrocyte catalase[17]. This inhibition of catalase by CO_2 was confirmed in a separate experiment (Wood, unpublished) in which guinea pig erythrocytes were mixed with 1% H_2O_2 in a Warburg respirometer. The rate of O_2 evolution in the presence of 15% CO_2 was ca. one-half that of the rate in air. This explanation for increased hemoglobin oxidation, however, is not supported by the GSH data, because H_2O_2 oxidizes hemoglobin only after GSH is fully oxidized[12]. Unless GSH peroxidase, as well as catalase, is inhibited by CO_2, GSH should disappear via oxidation in the GSH peroxidase reaction. Such inhibition is unlikely since GSH peroxidase is not inhibited by azide, a catalase inhibitor and is apparently not a heme enzyme[13].

Although catalase inhibition may play a minor role, the increase of methemoglobin may simply result from the shift of the hemoglobin = methemoglobin equilibrium due to the increased rate of autoxidation at low pH and inhibition of the methemoglobin reducing systems which normally maintain a low concentration of methemoglobin. Two of the pathways, NADPH-dependent methemoglobin reductase and nonenzymatic reduction by GSH, depend on hexose shunt activity, and are normally only of minor importance. As shown in Figure 2, inhibition of glucose-6-phosphate dehydro-

genase would bring about depletion of both NADPH and GSH. The relative stability of these parameters during the uncompensated phase of acidosis is consistent with the evidence that hexose monophosphate shunt activity is not a function of pH[18]. The reason for the sharp drop in both parameters after 7 days of 15% CO_2 is not apparent. Further studies are needed to elucidate the mechanism of these changes.

Since the hexose shunt redox systems did not appear to account for the methemoglobinemia during acute hypercapnia, we focused our attention on the third pathway, i.e., NADH-dependent methemoglobin reductase or "diaphorase." Inhibition by decreased pH of the Embden - Meyerhof Pathway of in vitro erythrocytes is well known [6, 18, 19]. Also, the ratio NAD+/NADH has been shown to increase during hypercapnia in rat brain[5] and erythrocytes[18, 19]. Using an experimental design identical to that of the present study, Jacey and Schaefer[9] showed that the NAD+/NADH ratio of guinea pig erythrocytes in vivo increases to a maximum value at one day and returns to control values at seven days of exposure to 15% CO_2. As seen in Figure 2, decreased NADH would limit activity of NADH dependent methemoglobin reductase. The changing NAD+/NADH ratio during hypercapnia reflects phosphofructokinase activity which at 1 day of 15% CO_2 exposure is 55% inhibited and remains ca. 30% inhibited after 7 days[10]. It seems evident, therefore, that the biphasic changes in methemoglobin concentration during the uncompensated phase of acidosis result mainly from pH inhibition of anaerobic glycolysis. The persistence of methemoglobin through the seventh day, after the NAD+/NADH ratio is back to normal, may result from the decreased GSH and G-6-P dehydrogenase activity during the seventh day.

Our observation that exposure is 3% CO_2 did not result in methemoglobin formation is somewhat surprising since 3% CO_2 produces respiratory acidosis at 1 day which is roughly equal to that at 7 days of 15% CO_2 where the methemoglobin level is significantly above that of control animals. This may result from differences in the magnitude pH gradient across the red cell membrane induced by 3% compared with 15% CO_2 exposure. Schaefer, et al.[20] found that the pH gradient across the red cell membrane was greater at 7 days exposure to 15% CO_2 than it was during early phases of acidosis. The maintenance of normal hemoglobin during exposure to 3% CO_2 may result from the maintenance of a smaller pH gradient (higher intracellular pH). The absence of methemoglobin formation during 3% CO_2 exposure is also consistent with the report of Jacey, Messier and Schaefer[11] that 3% CO_2 exposure does not alter the NAD/NADH ratio of guinea pig erythrocytes.

The results of this study are compatible with the clinical reports that methemoglobinemia is not associated with G-6-P dehydrogenase deficiencies or other disturbances of the NADP+/NADPH redox system, but is found in cases of NADH methemoglobin reductase deficiency. At present, however, it is not clear whether the changes in methemoglobin level result mainly from interference with the mechanisms which protect hemoglobin

from oxidation or from inhibition of the mechanisms which operate to reduce methemoglobin, or a combination of these factors.

SUMMARY

In guinea pigs exposed to 15% CO_2 in air for periods up to 7 days the methemoglobin concentration increased from 2.69% (controls) to 8.73% at 1 day and returned to 6.4% at 3 days and 6.2% at 7 days. The level of reduced glutathione and glucose-6-phosphate dehydrogenase activity did not show this biphasic change but decreased only at 7 days.

The time course of changes in methemoglobin concentration characterizes the uncompensated and compensated phases of respiratory acidosis and is correlated with biphasic changes in red cell pH, P_{50}, and "n" values of whole blood oxygen dissociation curves, and with erythrocyte NAD+/NADH ratios. The results of this study also provide an explanation for decreased oxygen capacity during hypercapnia.

Exposure to 3% CO_2 for periods up to seven days produced a mild acidosis but did not result in methemoglobin formation.

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13. ABSTRACT

Recent studies of chronic hypercapnia in guinea pigs and rats demonstrated a biphasic shift in the blood oxygen dissociation curve, a dimunition of heme-heme interaction and a significant decrease in the oxygen capacity of blood which was not due to decreased hemoglobin content. The latter finding suggested production of non-functional hemoglobin during hypercapnia. We measured the concentration of oxidized (met) hemoglobin and found an increase from control values of 2.69% to 8.73% at 1 day, 6.4% at 3 days and 6.2% at 7 days of 15% CO_2 exposure. The values are correlated with the biphasic changes in oxygen affinity (P_{50}) and heme-heme interaction (n) of blood oxygen dissociation curves during hypercapnia. Hemoglobin oxidation is enhanced by low pH and inhibition of red cell catalase by CO_2 resulting in increased oxidation by H_2O_2 may also be partially responsible for methemoglobin formation. The persistance of methemoglobin results from inhibition by low pH of the enzyme systems which convert methemoglobin to hemoglobin.

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